

THC Regulates Tearing via Cannabinoid CB1 Receptors

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PURPOSE. Aqueous deficiency dry eye (ADDE) is a chronic condition affecting millions, with symptoms ranging from a dry itchiness to blurred vision and accompanied by an increased risk of eye infections. ADDE typically arises from disorders of the lacrimal gland that produces tears necessary for eye lubrication. Cannabis users frequently report dry eye, but the basis for this is unknown. If the effects occur via the endogenous cannabinoid signaling system, then this may represent a novel mechanism for the regulation of tearing.

METHODS. We examined expression of cannabinoid CB1 receptors in the lacrimal gland using immunohistochemistry, Western blotting, and PCR and tested tetrahydrocannabinol (THC) regulation of tearing in wild-type and CB1-null mice.

RESULTS. We now report that CB1 receptors are expressed in the axons of cholinergic neurons innervating the lacrimal gland. Little if any staining is seen in lacrimal gland epithelial cells (acinar and ductal) or myoepithelial cells (MECs). Activation of CB1 receptors by THC or the cannabinoid agonist CP55940 reduces tearing in male mice. In female mice, THC has no effect, but CP55940 increases tearing. In both sexes, the effect of CP55940 is absent in CB1 knockout mice. CB1 mRNA and protein levels are approximately four- to fivefold higher in males than females. In male knockouts, THC increases tearing, suggesting that THC also acts through different receptors.

CONCLUSIONS. Our results suggest a novel, albeit sex-dependent, physiologic basis for the dry eye symptoms experienced by cannabis users: activation of neuronal CB1 receptors in the lacrimal gland reduces tearing.

Keywords: cannabinoid, CB1, 2-arachidonoylglycerol, THC, tearing, lacrimation, lacrimal gland

The lacrimal gland is an exocrine gland that produces the aqueous layer of the tear film.¹ Deficiencies in aqueous tears may lead to aqueous deficiency dry eye (ADDE). In humans, ADDE is a common condition that affects more than 10 million Americans² and can lead to blurry vision, eye pain, photophobia, itchy eyes, and red eyes.³ The annual burden of dry eye disease, which includes ADDE, on US health care is estimated to be in excess of \$3.5 billion; on average, afflicted individuals spend more than \$750 per year managing their symptoms.⁴ Many factors can influence chances of individuals developing the disorder, the most common of which are older age, female sex, use of antidepressant medication, and some autoimmune diseases.⁵ Lack of proper ocular lubrication can also lead to increased infections, chronic inflammation, affected vision, and corneal abrasions.⁶ Additionally, there is a feedback loop between the cornea and the lacrimal gland, whereby damage to the cornea results in tear reduction, further exacerbating the problem.⁷ Lacrimal gland secretions are under neuronal control, including sympathetic and parasympathetic innervation of the gland as well as brain regions such as the superior salivatory nucleus in the brainstem (reviewed in Dartt⁸).

Changes in the legal status of cannabis in Canada and many US states have been accompanied by increasing use of cannabis, but the ocular effects of cannabis consumption are still largely unknown due to limited research. Cannabis users commonly report dry eye,⁹ but the mechanism by which cannabis regulates tearing is unknown. Tetrahydrocannabinol (THC), the chief euphoric component of cannabis, has been shown to act by engaging an endogenous cannabinoid signaling system. This cannabinoid signaling system consists of the cannabinoid receptors CB1¹⁰ and CB2,¹¹ lipid messengers 2-arachidonoyl glycerol (2-AG) and anandamide,^{12,13} and enzymes that produce and metabolize these messengers (reviewed in Murataeva et al.¹⁴). The cannabinoid signaling system may also include several related receptors: GPR119,¹⁵ GPR55,¹⁶ GPR18,¹⁷ and perhaps TRPV1 since anandamide is a full agonist at this receptor.¹⁸ The cannabinoid signaling system also exists in the eye, where it has been shown to regulate ocular pressure^{19–21} and corneal wound healing.^{22–24}

Given the health burden that ADDE represents, investigating a novel mechanism of regulation of tearing might offer insights into some forms of ADDE or even indicate a

TABLE. Antibodies/Primary Labels Used in This Study

Target	Host	Source	Cat. No.	Lot No.	Concentration
CB1	Rabbit	Immunogenes	IMG-pAb001	BM01	1:300
Neurofilament H	Guinea pig	Synaptic Systems	171104	171104/1-2	1:300
β -actin	Chicken	Synaptic Systems	251006	1-1	1:300
β 3-tubulin	Guinea pig	Synaptic Systems	302304	302304/6	1:300
Choline acetyl transferase (ChAT)	Goat	Millipore	AB144P	2211015	1:150
Substance P	Guinea pig	Abcam	AB10353	GR3313766-1	1:300
Tyrosine hydroxylase	Guinea pig	Synaptic Systems	213104	213104/1-5	1:300
VAMP8	Rabbit	Synaptic Systems	104302	104302/1-17	1:300
Phalloidin-488	NA	ThermoFisher	A12379	1749905	1:300
CB1-L15	Guinea pig	Ken Mackie	NA	NA	1:500

NA, not applicable.

new therapeutic target for treatment of ADDE. We therefore initiated a series of experiments to clarify how cannabinoids regulate tearing, with results as reported below.

METHODS

Animals

Experiments were conducted at the Indiana University and Scripps Research Institute campus. All mice used for experiments were handled according to the guidelines of the respective university animal care committees and in accordance with the ARVO animal statement. Adult mice (both sexes, aged 3–8 months) were kept on a 12-hour (06:00–18:00) light dark cycle and fed ad libitum. C57BL/6J (C57), CD1, and CB1 knockout (KO) mice were kindly provided by the laboratory of Dr. Ken Mackie (Indiana University, Bloomington, IN, USA). Conventional CB1 null mice (CB1^{-/-}) were originally received from Dr. Catherine Ledent (Catholic University, Leuven, Belgium).²⁵

Measurement of Tearing

To measure tearing in mice, a phenol red thread (Zonequick; Oasis Medical, San Dimas, CA) was positioned at the rear corner of the right eye (the lateral cantus of the conjunctival fornix) for 10 seconds while the animal was held by a second experimenter. Tears discolor the threads for later quantification—more tearing results in a longer discolored portion of the thread. The length of the discolored portion can therefore be taken as measure of tearing, allowing comparison of experimental conditions to baseline conditions. For experiments that tested the effect of drug treatments (CP55940 and THC), animals were injected with drugs intraperitoneally (IP; 0.5 mg/kg; CP55940, 4 mg/kg [THC]) 1 hour before tear measurement.

Pilocarpine injections (7 mg/kg) were done subcutaneously in isoflurane-anesthetized mice. Animals were injected with either vehicle or CP55940 (IP) as above an hour before pilocarpine treatment. Animals were then anesthetized using isoflurane (3% induction, 1.5% maintenance). A baseline tearing volume was obtained as above but for 60 seconds (since the animals were immobile). Animals were then promptly injected with pilocarpine. After 4 minutes, a second 1-minute tearing measurement was taken, followed by another at 8 minutes. We found that 8 minutes was clearly sufficient to elicit an abundance of tears and used this time point for comparison. After this, animals were allowed to recover from isoflurane. After a period of monitoring, they were returned to the colony.

Immunohistochemistry

For immunohistochemistry, lacrimal glands were fixed in 4% paraformaldehyde for 45 minutes at 4°C, then placed sequentially in 10% and 30% sucrose in PBS overnight before being suspended in OCT (Thermo Fisher Scientific, Waltham, MA, USA) in a 15-mL plastic test tube, then submerged in cold (–80°C) methanol. Fixed eyes were sectioned on a Leica cryostat (Leica Microsystems, Wetzlar, Germany), and then sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific). Slides were blocked with BSA, followed by treatment with primary antibodies (in PBS, saponin, 0.2%) for 1 to 2 days at 4°C. See Table for a list of primary antibodies. In cases where secondary antibodies were required, a second staining with secondary antibody (~4 hours at room temperature (RT)) was done after washing off the primary antibody. Appropriate secondary antibodies were labeled with Alexa 488, Alexa 594, or Alexa 647 (Thermo Fisher Scientific). Slides were then mounted with mounting media containing 4',6-diamidino-2'-phenylindole dihydrochloride to visualize nuclei (Fluoromount; Sigma-Aldrich, St. Louis, MO, USA). Images were acquired with a Leica TCS SP5 (Leica Microsystems) or Zeiss LSM 780 confocal laser scanning microscopes (Zeiss, Jena, Germany). Images were processed using FIJI (available at <https://www.imagej.net>) and/or IMARIS Image Analysis Software (BITPLANE; Oxford Instruments, Abingdon, UK) and/or Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA) software. Images were modified only in terms of brightness and contrast.

Quantitative RT-PCR

Primers for cannabinoid receptor 1 were designed by using Primer-Blast (available at <https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>) and the corresponding mouse gene. Primer sequences are listed as follows:

CB1 primers set 1

S: 5'-CTG ATC CTG GTG GTG TTG ATC ATC TG-3'

AS: 5'-CGT GTC TGT GGA CAC AGA CAT GGT-3'

CB1 primers set 2

S: 5'-AGT CAT TGT CGC CTG CTC AA-3'

AS: 5'-TGG TCT CCA TGG CCA GAG TA-3'

CB1 primers set 3

S: 5'-GCC CAA ACC GCT TCA GTA G-3'

AS: 5'-ATT GAG CAG GCG ACA ATG ACT-3'

GAPDH

S: 5'-GAGAAACCTGCCAAGTATGATGAC-3'

AS: 5'-CAACCTGGTCTCAGTGTAGC-3'

Eyes were extracted and the lenses removed, and they were then immediately stored at -80°C . RNA was extracted with Trizol reagent (Ambion, Austin, TX, USA), and genomic DNA was removed with DNase 1 (NEB, Bethesda, MD, USA) following the manufacturer's instructions. Reverse transcription was performed using RevertAid (ThermoFisher Balkans, Vilnius, Lithuania). The cDNA products were then amplified using Sybr Green reagent (PwrSybr; Applied Biosystems, Carlsbad, CA, USA). Quantitative PCR was performed with a QuantStudio 7 thermocycler (Advanced Biosystems, Waltham, MA, USA). Primers for glyceraldehyde 3-phosphate dehydrogenase were also used as an internal control for each experimental condition with the threshold cycle set within the linear range (10-fold above baseline). Once the standard critical threshold (Ct) was set, the relative expression levels for genes were determined using the $\Delta\Delta\text{Ct}$ method.

Western Blot

Lacrimal glands were harvested and frozen on dry ice. Samples were homogenized in RIPA buffer (25 mM Tris, pH 8.0, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 0.5% CHAPS, 1 \times HALT protease and phosphatase inhibitor; Thermo Fisher Scientific, #78440) and centrifuged at 10,000 \times g for 10 minutes at 4°C . The supernatant was then collected and mixed with 4 \times sample buffer and incubated for 10 minutes at 65°C and run on a 4% to 12% NuPage gel (Thermo Fisher Scientific, #NP0323BOX). Following protein transfer, blots were stained (Revert Total Protein stain, #926-11011; Li-Cor Bioscience, Lincoln, NE) and scanned for total protein and then blocked in Li-Cor Blocking Buffer (Li-Cor Bioscience, #927-40000) for 60 minutes at room temperature. They were then incubated with guinea pig anti-CB1 L15 antibody (1:500; provided by the laboratory of Dr. Ken Mackie, Indiana University). This antibody targets the last 15 amino acids of the intracellular portion of the receptor. This was diluted in a mixture of Li-Cor Blocking Buffer and 1 \times PBS (1:1). Blots were incubated with primary antibody overnight at 4°C . The next day, blots were washed 4 \times 15 minutes at room temperature in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and then incubated in the blocking buffer above containing Li-Cor donkey anti-guinea pig IR800 antibody (#926-32411) for 1 hour at room temperature. Finally, blots were washed as above and scanned on a Li-Cor Odyssey near-infrared imager. Apparent molecular weights were determined using Chameleon molecular weight marker (Li-Core Bioscience, #928-60000). Band densities were calculated using FIJI software. Bands corresponding to CB1 were normalized to protein density (labeled with Ponceau S) using Excel software (Microsoft, Redmond, WA, USA). Prism 7 software (GraphPad Software, La Jolla, CA, USA) was used for graphing and statistical analysis.

Drugs

THC was obtained through the NIDA Drug Supply Program. CP55940, SR141716, and pilocarpine were obtained from Cayman Chemical (Ann Arbor, MI, USA). Drug concentrations were determined based on available literature for in vivo studies (e.g., Sain et al.²⁶ for CP55940).

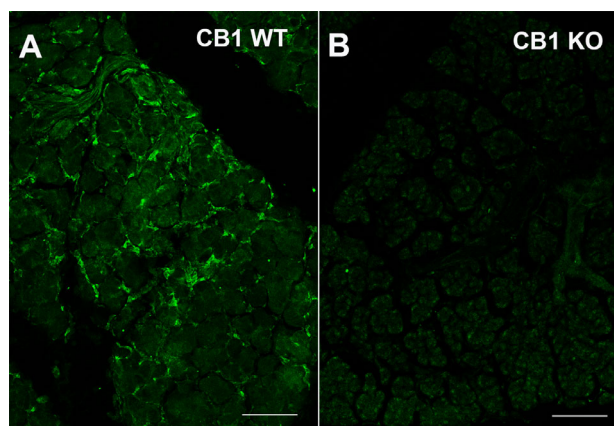


FIGURE 1. CB1 receptor protein is detected in the lacrimal gland. (A) Immunohistochemical staining in murine lacrimal gland slice shows CB1 protein expression in WT tissue. (B) CB1 staining is absent in CB1^{-/-} tissue. Scale bars: 50 μm .

RESULTS

CB1 Receptors Are Expressed in the Lacrimal Gland of the Mouse

Using immunohistochemistry, we tested CB1 receptor expression in the extraorbital lacrimal gland of the mouse (Fig. 1A). Specificity of the staining was confirmed in lacrimal glands from CB1 knockout mice (CB1^{-/-}) (Fig. 1B). We found that myoepithelial and acinar cells had no or little CB1 protein expression, while expression could be seen in small mesenchymally located cells (Figs. 2A, 2C). These may be immune cells, several of which are known to express cannabinoid receptors.^{27,28} In addition, CB1 protein has been found in neuronal axons. It has previously been reported that CB1 receptor is expressed in brain neurons and is important for neuronal function.²⁹ Lacrimal gland tearing is also under the control of different types of neurons that innervate the lacrimal gland.⁸ Coimmunostaining with the neuronal markers, such as neurofilament H (Fig. 3A) and β 3-tubulin (Fig. 3B), showed that CB1 receptor was expressed in a subset of neuronal processes. We also tested for colocalization of CB1 with markers for specific populations of neurons. The lacrimal gland is innervated by multiple neuronal inputs, including noradrenergic and substance P-positive fibers. We tested for colocalization with tyrosine hydroxylase (TH), an enzyme in the synthetic pathway for noradrenaline and so a marker for noradrenergic inputs. TH has been reported to be found near blood vessels in the lacrimal gland,³⁰ but we also saw TH around acini (Fig. 3C). Although CB1-positive processes were often closely juxtaposed to TH-positive processes, the staining did not colocalize. In Figure 3C, the two axons closely track one another but then clearly deviate. In Supplementary Figure S1, we show another example of this close but discrete protein expression, with orthogonal projections showing that the TH-positive staining corresponds to processes running alongside the CB1-positive process(es) (Supplementary Fig. S1). Substance P-positive fibers have been reported to be sparsely distributed in interlobular connective tissue and around ducts and blood vessels in the lacrimal gland.^{30,31} CB1 did not colocalize with substance P (Fig. 3D). We did, however, see coexpression of CB1 with choline acetyl transferase, a marker for cholinergic neurons (Fig. 3E).

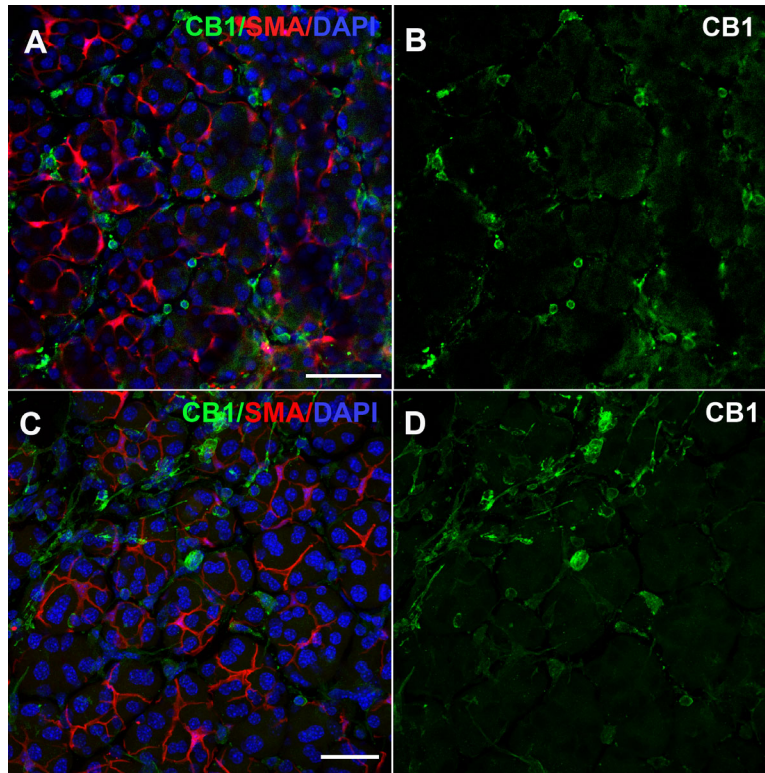


FIGURE 2. CB1 receptors are expressed in extra-acinar processes and cells. (A, B) Lacrimal CB1 protein expression (*green*) is seen outside acini, including small extra-acinar cell somata. Expression is not seen in green fluorescent protein (GFP)-expressing myoepithelial cells (“SMA”). (C, D) In a higher magnification image, CB1 is also visible in extra-acinar processes suggestive of neuronal axons. *Scale bars:* (A, B) 50 μm ; (C, D) 30 μm .

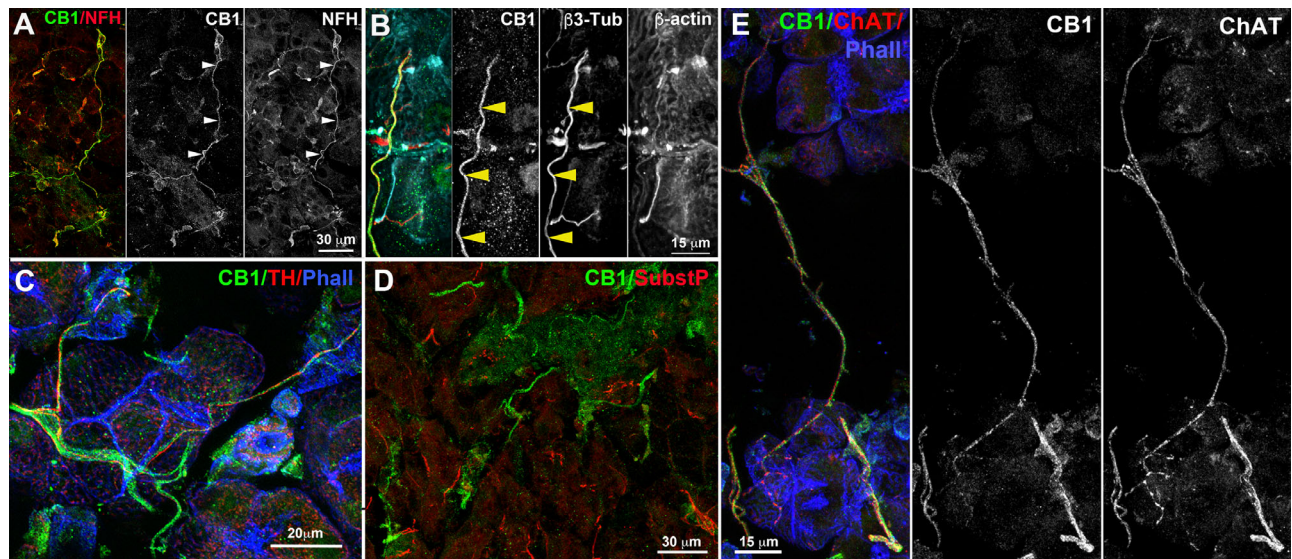


FIGURE 3. CB1 is expressed on neuronal axons. (A) CB1 (*green*) colocalizes with axonal marker neurofilament H (NFH; *red*). (B) Triple stain for CB1 (*green*), β 3-tubulin (*red*), and β -actin (*cyan*) shows colocalization with β 3-tubulin. (C) Staining for CB1 (*green*), tyrosine hydroxylase (TH; *red*), and phalloidin shows closely associated but discrete TH- and CB1-positive neuronal processes. (D) CB1 (*green*) and substance P (*red*) do not colocalize. (E) CB1 (*green*) colocalizes with cholinergic marker choline acetyl transferase (ChAT; *red*).

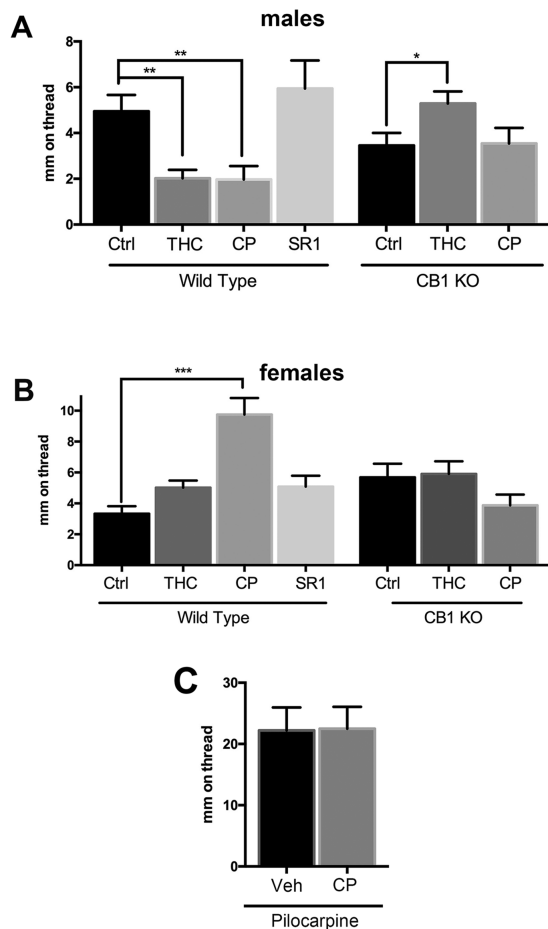


FIGURE 4. THC differentially alters basal tear volume in mice in a sex-dependent manner via activation of cannabinoid CB1 receptors. **(A)** In male wild-type mice, THC (4 mg/kg, IP) and the CB1 receptor agonist CP55940 (CP; 0.5 mg/kg, IP) lowered tear volume while the CB1 receptor antagonist SR141716 (SR1; 4 mg/kg, IP) did not. In CB1 knockouts, THC did not reduce tearing but instead increased tearing while CP55940 was without effect. $*P < 0.05$, $**P < 0.01$, one-way ANOVA with Dunnett's post hoc test versus control. **(B)** Female mice saw no effects from THC treatment (4 mg/kg, IP), but an increase in tearing after treatment with CP55940 (0.5 mg/kg, IP). SR141716 (4 mg/kg, IP) did not alter tearing. In CB1 knockouts, neither THC nor CP55940 affected tearing. $***P < 0.005$, one-way ANOVA with Dunnett's post hoc test versus control. **(C)** Pilocarpine (7 mg/kg, subcutaneously) elicited tearing in male mice as expected, but no difference in tearing was seen between animals injected with vehicle versus CP55940 (0.5 mg/kg) an hour prior to pilocarpine. NS, unpaired *t*-test.

THC Differentially Alters Basal Tear Volume in Mice in a Sex-Dependent Manner via Activation of Cannabinoid CB1 Receptors

Tearing is subject to regulation by multiple internal and external factors. *Basal lacrimation* is the tearing present under normal conditions, while lacrimation induced by an irritating stimulus is referred to as *reflex tearing*. We tested the consequence of THC treatment on basal tearing since this most closely approximates the conditions under which cannabis users would experience altered tearing. Male mice treated with THC (4 mg/kg, IP) showed a significant reduction in tear volume relative to wild-type (WT) controls (Fig. 4A; WT tear volume [mm on phenol red thread

\pm SEM]: 5.0 ± 0.7 , $n = 18$; THC treated: 2.0 ± 0.4 , $n = 18$; $**P < 0.01$, one-way ANOVA with Dunnett's post hoc versus WT control), consistent with the reduced tearing seen in cannabis-exposed human subjects.⁹ Similarly, the potent CB1 receptor agonist CP55940 (0.5 mg/kg) also lowered tearing volume in WT (Fig. 4A; CP55940 [mm \pm SEM]: 2.0 ± 0.6 , $n = 12$; $**P < 0.01$, one-way ANOVA with Dunnett's post hoc versus WT control). Treatment of the male CB1 knockout mice with the CB1 receptor agonist CP55940 (Fig. 4A; CB1 KO control [mm \pm SEM]: 3.5 ± 0.5 , $n = 17$; CP55940 in CB1 KO: 3.5 ± 0.7 , $n = 14$) did not alter basal tearing. Notably, we found that THC application in CB1 receptor knockout mice increased tearing, suggesting that THC may have a second target besides CB1 receptors (Fig. 4A; THC in CB1 KO: 2.0 ± 0.4 , $n = 18$, $*P < 0.05$, one-way ANOVA with Dunnett's post hoc versus CB1 KO control). This suggests that in male WT mice, the inhibitory CB1-mediated effect of tearing reduction is dominant. The CB1 receptor antagonist SR141716 (4 mg/kg) did not alter tearing in male WT mice (Fig. 4A; SR141716 [mm \pm SEM]: 5.9 ± 1.2 , $n = 14$, NS, as above). In contrast to our findings in males, female mice treated with THC did not see a change in basal tear volume (Fig. 4B; WT tear volume [mm \pm SEM]: 3.3 ± 0.5 , $n = 16$; THC treated: 5.0 ± 0.5 , $n = 18$, NS; one-way ANOVA with Dunnett's post hoc versus WT control). However, the CB1 receptor agonist CP55940 substantially increased tearing (Fig. 4B; CP treated: 9.8 ± 1.1 , $n = 16$; $P < 0.005$, one-way ANOVA with Dunnett's post hoc versus WT control). Treatment of the female CB1 knockout mice with the CB1 receptor agonist CP55940 (Fig. 4B; CB1 KO control [mm \pm SEM]: 5.7 ± 0.9 , $n = 20$; CP55940 in CB1KO: 3.9 ± 0.7 , $n = 16$, NS, one-way ANOVA with Dunnett's post hoc versus KO control) did not alter basal tearing. As in males, the CB1 receptor antagonist did not alter tearing in females (Fig. 4B; SR141716 in WT females: 5.1 ± 0.7 , $n = 16$). The results suggest that in females, activation of CB1 receptors increases tearing and that there is a pronounced sex dependence of CB1 regulation of tearing. In addition to basal and reflex tearing, lacrimation is also under the influence of sympathetic/parasympathetic regulation. Activation of the parasympathetic nervous system increases tearing via acetylcholine activation of muscarinic M3 receptors,^{8,32} an effect that is mimicked by the muscarinic agonist pilocarpine. We tested whether CB1 receptor activation by CB1 receptor agonist CP55940 in males also reduced pilocarpine-induced tearing. As expected, subcutaneous injection of pilocarpine increased tearing, but CP55940 (0.5 mg/kg, IP) did not have any effect on pilocarpine-induced tearing (Fig. 4B; pilocarpine [7 mg/kg] after vehicle [mm \pm SEM]: 22.2 ± 3.8 , $n = 10$; pilocarpine after CP55940 [0.5 mg/kg]: 22.5 ± 3.6 , $n = 8$, NS by unpaired *t*-test). Taken together, our results indicate that CB1 activation reduces basal tearing but not tearing induced by activation of the parasympathetic system.

Males Express Higher Levels of CB1 mRNA and Protein Than Females

Since we observed a sex dependence of CB1 regulation of tearing, we tested whether there might be a differential expression of CB1 in males versus females. We used quantitative PCR to examine levels of CB1 in the lacrimal gland, finding that levels of CB1 message were approximately fourfold lower in females than in males (Fig. 5A, relative expression levels [value \pm SEM, normalized to

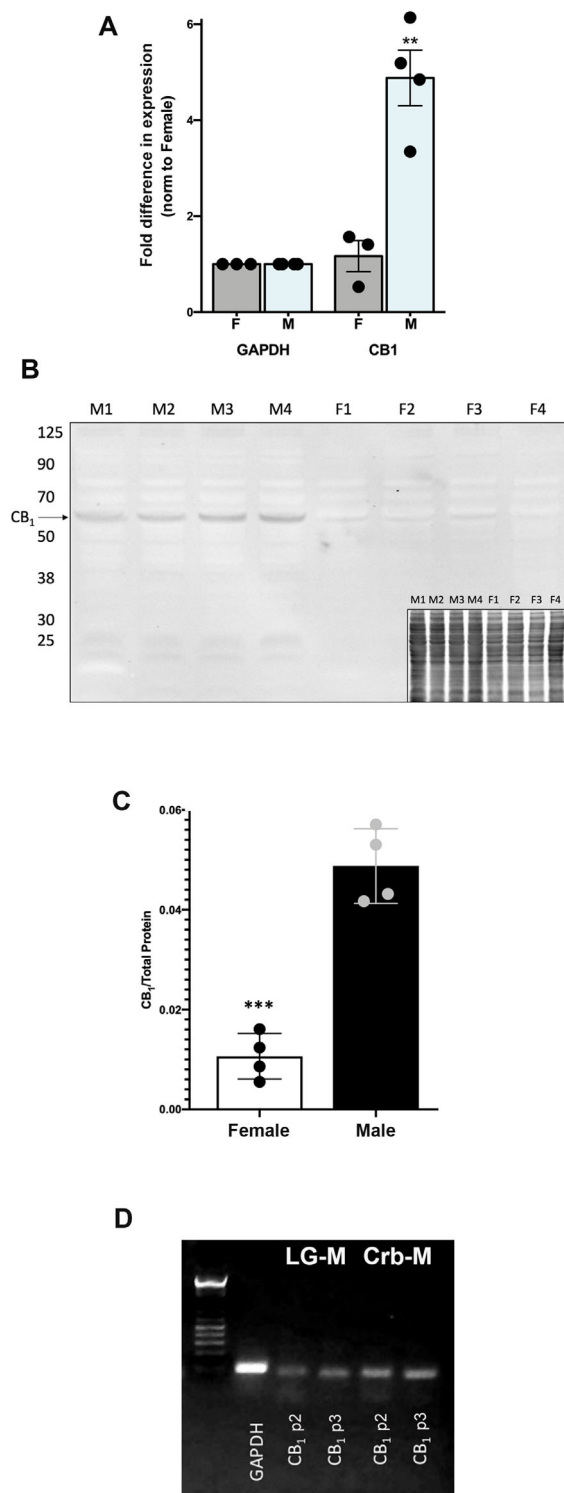


FIGURE 5. CB1 mRNA and protein are expressed at higher levels in male lacrimal gland. (A) Quantitative RT-PCR shows that message for CB1 receptors is approximately five times more abundant in male than in female mice. $n = 3, 4$, $^{***}P < 0.01$ by unpaired t -test. (B) Western blot for CB1 protein shows CB1 expression in lacrimal glands of four male and four female mice. Ponceau S staining is shown in *inset*, *bottom right*. (C) Quantification of CB1 protein expression (shown in C) in males and females (CB1 protein level relative to whole protein). $n = 4$, $^{***}P < 0.0001$ by unpaired t -test. (D) RT-PCR shows the presence of mRNA for CB1 in lacrimal gland (LG) and cerebellum (Crb) of a male mouse, using two different probe sequences (p1, p2 in figure). Glutaraldehyde 3-phosphate dehydrogenase (GAPDH) is included as a control.

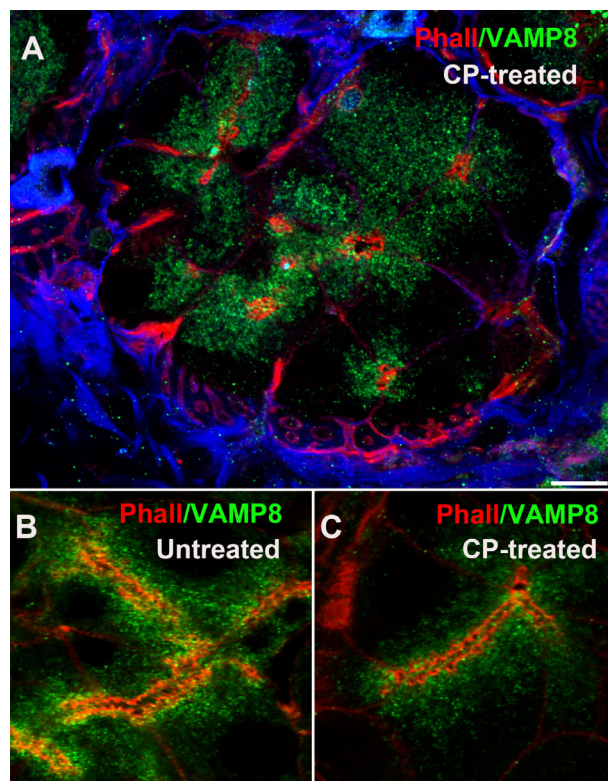


FIGURE 6. CB1 activation does not alter distribution of acinar vesicles in the lacrimal gland. (A) Staining for VAMP8 (*green*) is seen in acinar cells. Staining is polarized within acini, associated with intra-acinar ducts labeled with phalloidin (*red*), a marker for F-actin. (B, C) Higher magnification image of untreated lacrimal gland has similar staining pattern to that of a CP55940-treated (0.1 mg/kg, IP, 1 hour) animal. Lacrimal glands are from male mice. Scale bars: 10 μ m.

female]: females: 1.2 ± 0.3 , $n = 3$; males: 4.9 ± 0.6 , $n = 4$; $P < 0.01$ by unpaired t -test). This difference in CB1 expression may account for the sex-dependent effect of CB1 activation on tearing. In addition, we made use of Western blot for CB1 protein in lacrimal gland homogenate. Comparing levels of CB1 protein relative to whole protein, CB1 protein was found to be expressed at a higher level in males than in females (Figs. 5B, 5C; CB1 expression relative to total protein [\pm SEM]: males: 0.05 ± 0.004 , $n = 4$; females: 0.01 ± 0.002 , $n = 4$; $P = 0.0001$ by unpaired t -test). In addition, we used RT-PCR to assess expression of CB1 mRNA in lacrimal gland and cerebellum in male mice, since cerebellum has strong expression in synaptic terminals innervating Purkinje cells³³ (Fig. 5D).

Distribution of VAMP8-Positive Vesicles Is Unaltered by Treatment With CB1 Agonist CP55940

One potential mechanism of action for CB1 is a change in the distribution of vesicles within acini. We examined whether CB1 receptor activation altered the distribution of vesicle-associated membrane protein 8 (VAMP8), a SNARE protein involved in exocytosis in acinar cells such as those found in the lacrimal gland.³⁴ As shown in Figure 6A, VAMP8 showed the expected polarized distribution within acini, associated with intra-acinar ducts labeled with phalloidin, a marker for

F-actin. However, there was not an appreciable difference between the VAMP8 expression patterns in lacrimal glands from untreated versus CP55940-treated (0.1 mg/kg as above) males (Figs. 6B, 6C).

DISCUSSION

Cannabis users frequently report dry eye as an undesirable side effect. To our knowledge, only one prior study has explored the relationship between cannabis and tearing; nearly 50 years ago, Hepler et al.⁹ reported that cannabis reduces tearing in humans. The underpinning of this reduction has not been the subject of active study until now, despite the health burden of ADDE. In this study, we report three principal findings. First, cannabinoid CB1 receptors are present in the mouse lacrimal gland with notable expression in cholinergic axons of neurons innervating the lacrimal gland. Second, we find that THC, the chief euphoric constituent of cannabis, reduces tearing via activation of cannabinoid CB1 receptors in male mice, most likely via changing neuronal signaling. Third, the effect of CB1 activation is highly sex dependent: CB1 activation in female mice increases tearing, a difference that may arise from higher expression of CB1 in the male lacrimal gland.

The cannabinoid signaling system is complex, with multiple cannabinoid receptors, lipid messengers, and enzymes that produce and metabolize these lipid messengers. CB1 receptors are the predominant cannabinoid receptors in the central nervous system (CNS) and peripheral nervous system,^{35,36} and it is likely that CB1 receptor-expressing lacrimal gland nerves mediate the reduction in tearing reported here. CB1 receptors are G protein-coupled receptors (GPCRs) that largely signal via $G_{i/o}$ G proteins. In neurons, the CB1-mediated inhibition of calcium channels is coupled to a reduction in neurotransmitter release.³⁷ Inhibition of neurotransmitter release is a common theme of CB1 function in the CNS.²⁹ Therefore, cannabinoid CB1 receptors may regulate tearing by modulating the release of neurotransmitters. As $G_{i/o}$ -coupled GPCRs, CB1 receptors also generally inhibit adenylyl cyclase, reducing cAMP levels and activate ERK signaling.^{38,39} As noted earlier, tearing is controlled by multiple mechanisms. One major source of lacrimal regulation is the parasympathetic system: it has been established that parasympathetic activation elicits tearing³² via muscarinic M3 receptors.⁴⁰ We showed that while CB1 is expressed in cholinergic neurons, pilocarpine-induced tearing was not reduced by CB1 receptor agonist treatment. These results are similar to what was found in the salivary gland, a related secretory structure also under neuronal control. McConnell et al.⁴¹ showed that THC reduced salivary flow but did not impact pilocarpine- or acetylcholine-stimulated salivary flow. They did, however, find evidence for a reduction of ACh release. They concluded that THC reduced salivary flow via inhibition of ACh release. A similar CB1-dependent arrangement may be the case here.

Thus, our data indicate that CB1 activation reduces basal tearing in male mice. We have previously seen evidence of tonic CB1 activity in regulation of ocular pressure.⁴² If tearing were also under partial tonic inhibition by CB1 receptors, then reversing this might result in enhanced tearing, a potentially desirable effect. However, we did not see evidence of tonic CB1 receptor activity, since the CB1 antagonist SR141716 did not increase tearing and the baseline tearing

for CB1 knockouts was not different from WT controls. In female mice, however, the picture is very different. THC has no significant effect on tearing in females, but CB1 activation substantially increases tearing, the opposite of what is seen in males. We found that expression of mRNA and protein for CB1 receptors was approximately four- to fivefold higher in males than females, so it is possible that in females, the regulation of tearing occurs outside of the lacrimal gland.

Significantly, it is likely that THC acts at two targets, since THC treatment in CB1 receptor knockout mice increased tearing. THC activates not only the CB1 receptor but also other receptors, including CB2, GPR18, GPR119, and some peroxisome proliferator-activated receptor (PPARs).⁴³ Given the number of receptors that are engaged by THC, it is not unexpected that THC might have multiple effects on the same system via different effectors. For example, in the anterior eye, we found that THC lowered pressure by activating a combination of CB1 and GPR18 receptors.⁴² In the same study, we reported that the related phytocannabinoid cannabidiol had opposing effects on ocular pressure. It will be important to determine this second target of THC; if it is a receptor, then that receptor would be of considerable interest in the context of dry eye syndrome since a selective agonist for that target would be expected to enhance tearing.

Since it is likely that CB1-based cannabinoid signaling is taking place in the lacrimal gland—particularly in males—a final consideration is what other components of the cannabinoid signaling system might be active there. Of the two structurally related endogenous lipid messengers that have been identified, 2-AG and arachidonoyl ethanolamide (anandamide), Matsuda et al.⁴⁴ detected the presence and synthesis of the anandamide in homogenate of porcine lacrimal gland. Cannabinoid signaling in the lacrimal gland may therefore involve anandamide, but this does not rule out a potential role for 2-AG.

In summary, we find that the dry eye symptoms frequently reported by cannabis users are likely due to THC activation of cannabinoid CB1 receptors in the lacrimal gland. Future studies are necessary to elucidate a detail the mechanisms of cannabinoid signaling in the lacrimal gland.

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